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Abstract: Currently, there are several treatments for osteoporosis however; they all display some sort of limitation and/or side effects making the need for new treatments imperative. We have previously demonstrated that NMP is a bioactive drug which enhances bone regeneration in vivo and acts as an enhancer of bone morphogenetic protein (BMP) in vitro. NMP also inhibits osteoclast differentiation and attenuates bone resorption. In the present study, we tested NMP as a bromodomain inhibitor and for osteoporosis prevention on ovariectomized (OVX) induced rats while treated systemically with NMP. Female Sprague-Dawley rats were ovariectomized and weekly NMP treatment was administrated 1 week after surgery for 15 weeks. Bone parameters and related serum biomarkers were analyzed. 15 weeks of NMP treatment decreased ovariectomy-induced gained weight in average by 43% and improved bone mineral density (BMD) and bone volume over total volume (BV/TV) in rat femur on average by 25% and 41% respectively. Moreover, mineral apposition rate and bone biomarkers of bone turnover in the treatment group were at similar levels with those of the Sham group. Due to the function of NMP as a low affinity bromodomain inhibitor and its mechanism of action involving osteoblasts/osteoclasts balance and inhibitory effect on inflammatory cytokines, NMP is a promising therapeutic compound for the prevention of osteoporosis.

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Original Full Length Article

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ABSTRACT

Currently, there are several treatments for osteoporosis however; they all display some sort of limitation and/or side effects making the need for new treatments imperative. We have previously demonstrated that NMP is a bioactive drug which enhances bone regeneration *in vivo* and acts as an enhancer of bone morphogenetic protein (BMP) *in vitro.* NMP also inhibits osteoclast differentiation and attenuates bone resorption.

In the present study, we tested NMP as a bromodomain inhibitor and for osteoporosis prevention on ovariectomized (OVX) induced rats while treated systemically with NMP. Female Sprague- Dawley rats were ovariectomized and weekly NMP treatment was administrated 1 week after surgery for 15 weeks. Bone parameters and related serum biomarkers were analyzed. 15 weeks of NMP treatment decreased ovariectomy-induced gained weight in average by 43% and improved bone mineral density (BMD) and bone volume over total volume (BV/TV) in rat femur in average by 25% and 41% respectively. Moreover, mineral apposition rate and bone biomarkers of bone turnover in the treatment group were at similar levels with those of the Sham group.

Due to the function of NMP as a low affinity bromodomain inhibitor and its mechanism of action involving osteoblasts/osteoclasts balance and inhibitory effect on inflammatory cytokines, NMP is a promising therapeutic compound for the prevention of osteoporosis.

Key Words:

Epigenetic, NMP, osteoporosis, bromodomain, BET proteins, BRD, estrogen deficiency, ovariectomy

1. INTRODUCTION

Osteoporosis is a skeletal disorder characterized by compromised bone strength that predisposes to increased risk of fracture. It is most often caused by an increase in bone resorption that is not sufficiently compensated for by a corresponding increase in bone formation [1].

Osteoporosis poses a significant public health issue. It is estimated that over 200 million are affected by osteoporosis worldwide. The number of women with osteoporosis and subsequent fractures are bound to rise as the elderly population increases [2]. Advanced age and early menopause are the best predictors of osteoporosis, but other factors such as; low body weight, diseases, treatments, family history of osteoporosis and inactive lifestyle increase susceptibility to fractures [3]. The cost of osteoporosis-related fractures to the economy is enormous, predicted to escalate to $131.5 billion by 2050 [4]. In Europe alone 22 million women and 5.5 million men are estimated to have osteoporosis [5]. Because of the vast medical and socioeconomic challenges that osteoporosis present worldwide; the need for new treatments is both imperative and pressing.

At the moment, some of the most used therapies for osteoporosis include, but are not limited to, bisphosphonates, parathyroid hormone (PTH), and selective estrogen receptor modulators (SERMs). Over the last decade, as bisphosphonates became an established treatment for osteoporosis, potential side effects of patients under this therapy have been reported. The most common includes atypical subtrochanteric or femoral shaft fractures and osteonecrosis of the jaw, however, at very low frequency [6]. Also, severe suppression of bone turnover is caused by bisphosphonate usage [7]. Instead of preventing bone resorption, the opposite strategy for treating osteoporosis is the application of anabolic substances such as PTH (1-34) applied in intermittent regime [8]. SERMs have been developed and evaluated for osteoporosis

treatment and prevention, including bazedoxifene, lasofoxifene, droloxifene, idoxifene, ormeloxifene, ospemifene, and arzoxifene [9]. Nevertheless, the most crucial feature to define the clinical efficacy of a SERM is generally considered to be endometrial safety [10].

A more recent trend is epigenetic drug discovery showing great potential for new therapies [11]. Epigenetics refers to transmissible changes in gene expression that does not involve changes to the underlying DNA sequence. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change [12]. BET (bromodomain and extraterminal domain) proteins are a group of epigenetic regulators. They were shown to serve as scaffolds for molecular complexes at recognized acetylated histone sites to regulate chromatin accessibility to transcription factors and RNA polymerase[13]. They are considered potential therapeutic targets in many distinct diseases. The role of epigenetics in osteoporosis has just starting to be studied. However, it is gradually being postulated as a key concept, as epigenetic mechanisms are involved in the interactions between the genome and the environment. The exact relationship between epigenotypes and disease phenotypes is still to be elucidated; it is known that epigenetic marks change during aging, including a global decrease in the abundance of 5- methylcytosines and some histone modification [14]. Since osteoporosis is an age-related disease, it could be speculated that those age-related changes in epigenetic marks participate in the pathophysiology of the disease [15]. Recent studies demonstrated JQ1 (a bromodomain inhibitor developed by *the Structural Genomic Consortium*) to suppress inflammation by a reduction of the inflammatory cytokine release, bone destruction by the inhibition of osteoclast maturation and bone formation by the inhibition of

osteoblast maturation [15, 16]. The latter activity makes its use for the treatment of osteoporosis questionable.

Recently, it was discovered that NMP also exhibits some affinity to bromodomains [17]. Over the last years we showed that N-methyl pyrrolidone (NMP), a small water soluble molecule used as a constituent in FDA-approved medical devices plays a significant role in the osteoblast and osteoclast differentiation [18, 19]. Indeed, NMP enhances BMP-2-induced osteoblast differentiation and bone regeneration and disrupts osteoclast differentiation and bone resorption. Together these results suggest that NMP might act as clinically applicable bromodomain inhibitor and could be used for the prevention or treatment of osteoporosis.

To that end we tested NMP for its ability to inhibit bromodomain binding for a variety of BET proteins and for the prevention of osteoporosis in ovariectomized (OVX) animals, a well-established animal model mimicking menopause in women and simulating osteoporosis.

2. Materials and methods

2.1 AlphaScreening Assay.

AlphaScreening Assay was performed using recombinant bromodomains and bromodomain ligands or recombinant BET bromodomains and BET Ligand from BPS Bioscience (San Diego, USA). The AlphaScreening signal from the assay is correlated with the amount of bromodomain/BET ligand binding to the bromodomain. AlphaScreening signal was measured using EnSpire Alpha 2390 Multilabel reader (Perkin Elmer).

Binding experiments were performed in duplicate. AlphaScreening data were analyzed using the computer software, Graphpad Prism. In the absence of the compound, the AlphaScreening signal (A_t) in each data set was defined as 100% activity. In the absence of the bromodomain/BET Ligand, the AlphaScreening signal (A_b) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity = $[(A- A_b)/(A_t - A_b)]\times100$, where A= AlphaScreening signal in the presence of the compound, A_b = AlphaScreening signal in the absence of the bromodomain/BET Ligand, and A_t = AlphaScreening signal in the absence of the compound. The percent inhibition was calculated according to the following equation: % inhibition = 100 - % activity. Values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation $Y=B+(T-B)/1+10^{((LogEC50-X)\times Hill\ Slope)}$, where Y=percent activity, B=minimum percent activity, T=maximum percent activity, X= logarithm of compound and Hill Slope=slope factor or Hill coefficient. The IC50 value was determined by the concentration causing a half-maximal percent activity.

2.2 Assay of ALP activity and ALP staining

Alkaline phosphatase activity was measured as a marker of osteoblastic differentiation. C2C12 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin). For the experiments, the cells were plated one day before treatment and treated with BMP2 in the presence or absence of different

agents. C2C12 cells were seeded at a density of 5×10^4 cells/cm^2 in 24-well plates (*n* = 3 per group) for ALP staining or in 96-well plates (n=4 per group) for ALP activity. One day later, cells were treated as indicated in the figure, and incubation was continued for 5 more days. After 5 days of incubation, medium was removed, and cells were washed with PBS and then scrapped in buffer A (0.56 M 2-amino-2-methyl-1-propanol). The pellets were then homogenized for 10 s. After centrifugation, supernatant was collected and used for ALP assay using p-nitrophenylphosphate as a substrate. The protein content of the lysates was measured using Bradford protein assay reagent (Bio-Rad). Experiments were performed independently in triplicate. To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were stained as described in [20]. Images of stained cells were captured with a CDD camera.

2.3 Osteoporosis Rat Model and Treatments

15 weeks old healthy female Sprague-Dawley (SD) rats (wt. 230 ± 10 g) were obtained from Charles River laboratories. The rats were adapted to laboratory environment for 2 week before the experiment. In 3 independent experiments a total of 30 animals were used. The acclimatized rats underwent either bilateral laparotomy (Sham Veh, N_{total}=10) or bilateral ovariectomy (OVX, N_{total}=20). One week after recovering from surgery, the OVX rats were divided into 2 groups: OVX with vehicle (OVX Veh, N_{total}=10) and OVX with NMP (OVX NMP, 1/3 of LD50 = 105µl/100g/week, equals an overall concentration of 10.5 mM, N_{total}=10). Treatment via intraperitoneal injection was initiated 1 week after OVX and lasted for 15 weeks. The body mass of each rat was monitored weekly, and the administered dose was

adjusted accordingly. All animal procedures were approved by the Animal Ethics Committee of the local authorities (Canton Zurich, 40/2012). Whole blood sample was collected via abdominal aorta puncture immediately following sacrifice by CO_2 asphyxiation. Then, a serum specimen was harvested after centrifugation (2000 rpm for 20 min). Samples were stored at −80°C until further testing and analysis. Femurs were dissected and the adherent tissue removed before placing the samples in 70 % ethanol and later used for bone mineral density (BMD) measurement and trabecular microarchitecture analysis. Liver tissues were also removed and fixed and embedded in paraffin (Sophistolab AG, Muttenz, Switzerland) staining with H&E and analyzed for pathological changes (toxicity).

2.4 Microcomputed Tomography (μCT) Analysis.

The rat femur samples were measured with a cone-beam microCT (μCT 100, SCANCO MEDICAL AG, Brüttisellen, Switzerland) at a resolution of 14.8 μm. The reference point was used to define the region of interests (ROI) and the bone was automatically segmented, based on its gray scale value in the CT slices. 200 slices were evaluated for every sample (volume of interests: VOI). The three-dimensional images were reconstructed with the purpose of visualization and display. After analyzing the VOI, morphometric bone parameters, including bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th) were obtained. The VOI analysis was performed blindly by the same operator. Cortical region of distal femur was also analyzed. The bone was automatically segmented, based on its gray scale value in the CT slices. The region of interest (cortical bone) was then selected by a contour. The samples were

segmented based on its gray scale values in the CT slices. 88 slices were evaluated for every sample.

2.5 Dynamic Histomorphometry

To compare the dynamic rate of bone formation in Sham and OVX rats, animals were injected two times with calcein (25 mg/kg, i.p.) 10 days apart. The animals were sacrificed 3 days after the second injection and undecalcified calvarias were embedded in methyl methacrylate. Coronal Sections (5 µm thickness) of the paraxial mesoderm of the calvarial bones were then prepared and three subsequent sections were analyzed for each animal. New bone formation was assessed by fluorescence microscopy of calcein green using a fluorescein isothiocyanate filter. The mineral apposition rate (MAR) was calculated on the basis of interlabeled width between the calcein double labelling.

2.6 Histology

Paraffin sections for histological analysis of bone quality were prepared and stained with hematoxylin and eosin (H & E) as well as Goldner Trichrome as described earlier [19]. The sections were examined for changes in the bone marrow and staining was visualized with a Leitz Dialux20 microscope and images captured using a Leica camera.

2.7 Serological Analysis of Bone Markers

Serum markers were analyzed to monitor the treatment effect on bone physiology. Serum concentration of Estradiol, Osteocalcin (Takara), Cross Linked C-Telopeptide of Type I Collagen (CTXI) and Protocollagen I N-Terminal Propeptide (PINP) (Uscn Life Science) were measured according to the manufacturer's instructions.

2.8 Statistical analysis

All statistical analysis were performed with IBM SPSS statistics 22. Data from all parameters were normally distributed (Shapiro-Wilk test). Results are expressed as the mean ± SD and were compared by ANOVA and Student's t-test. Results were considered significantly different for $P<0.05$.

3. Results

3.1 Effect of NMP on the binding ability of bromodomains and BET bromodomains

Bromodomain is an evolutionarily conserved, ~110 amino acid motif comprised of four left-handed, antiparallel α-helices [21]. The discovery of the drugability of bromodomains led to the screening of and identification of small molecules which can be used as bromodomain inhibitors in different epigenetic mechanisms. To assess the possibility that NMP might act as a bromodomain inhibitor, we used an AlphaScreen assay format to determine the effect of NMP on the binding ability of different recombinant human bromodomains and recombinant human BET bromodomains. By using recombinant human BET, NMP shows slightly higher inhibitory effect compared to bromodomains proteins. Indeed, NMP inhibits the

binding activity of BRD2-BD1BD2 and BRD4-BD1BD2 by 78% and 71% respectively (**Fig.1A**). The pan-BET inhibitor JQ1 was used as control. AlphaScreen dose response experiments carried out against BDR2 and BRD4 gave rise to millimolar half-maximum inhibitory concentration (IC50) values for NMP and nanomolar for JQ1 (**Fig.1B**). Also, NMP+BMP combination resulted in higher ALP activity and staining compared to BMP alone and JQ1+BMP (**Fig.1C**). Since JQ1 was shown to suppress inflammation by a reduction of the inflammatory cytokine release and bone destruction by the inhibition of osteoclast maturation, we assumed that alongside the *in vitro* effect, NMP could have an *in vivo* effect on bone remodeling. For that we decide to test the effect of NMP on ovariectomy-induced bone loss.

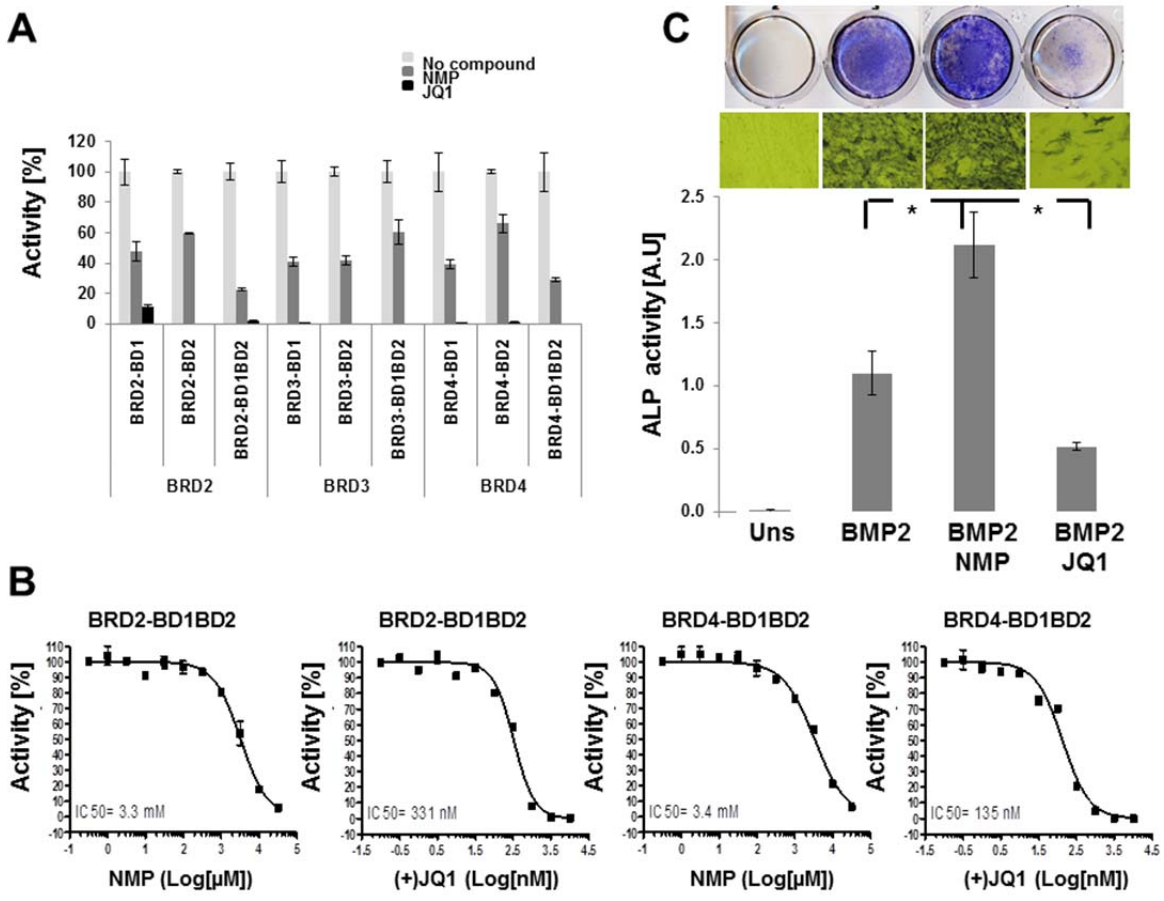

Figure 1: NMP Effect on the binding ability of bromodomains and BET bromodomains: Figure 1A demonstrates the effect of NMP on the binding ability of recombinant BET bromodomains using the AlphaScreening assay where the signal from the assay is correlated with the amount of bromodomain/BET ligand binding to the bromodomain. Figure 1B shows the determination of millimolar half-maximum inhibitory concentration (IC50) for NMP and nanomolar for (+) JQ1 on BRD2 and BRD4 binding activity. (C) Effects of NMP and (+) JQ1 on BMP-2-induced ALP

activity and ALP staining in C2C12 cells are shown (upper panel overview of the cell culture well, lower panel higher magnification, lowest panel corresponding ALP activity).

3.2 Effects of estrogen deficiency (OVX) on body weights and estradiol levels

To investigate the effect on NMP treatment on ovariectomy induced bone loss, we subjected rats to ovariectomy (OVX) and weekly injections of 1/3 of the LD50 level for NMP based on the weight of the animals [22]. All animals tolerated the procedure well and did not show any adverse effects. From the 30 animals used in 3 independent experiments, one animal of the 3rd round OVX Veh group died in the second week of unknown reasons. Therefore, we report here on Sham Veh (N_{total}=10), OVX Veh (N_{total} =9) and OVX NMP (N_{total} =10). A Sham NMP group was included in pretests but showed no significant difference compared to the Sham Veh group (Supplemental figure 1). In order to confirm that the ovariectomy was effective we measured the body weight of the animals weekly. There was no significant difference in the mean body weight initially in each group however, after 15 weeks of treatment ovariectomy promoted an important increase in body mass over time compared to the Sham group (**Fig.2A**). OVX group on the other hand treated with NMP gained significantly less weight than the OVX Veh group. Over the course of 15 weeks the animals gained weight more rapidly the first 3 weeks and then steadily increased over time still keeping a substantial variance between the animal groups at all time. Weekly NMP injection to rats for the duration of 15 weeks resulted in no toxicological changes in any of the clinical signs; body weight changes and liver damage (Supplemental figure 2). Thus, under the present experimental conditions, the No Observable Adverse Effect Level (NOAEL) of NMP was assumed to be 1ml/kg/week for female rats.

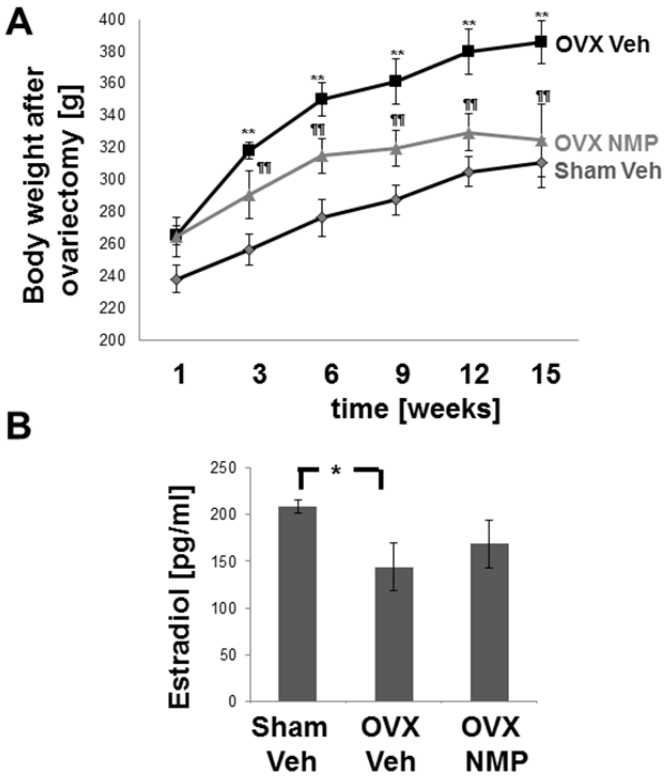

Figure 2: Body weight changes in the OVX model of osteoporosis: OVX rats received no treatment (OVX Veh), OVX NMP rats were treated with 105µl/100g/week of NMP for 15 weeks. Sham group was not treated with NMP (A) and estradiol serum level was compared between groups (B). Values are mean ± SD; error bars in the figure are presented as SD.

Estrogen is an antiresorptive agent which interacts with osteoblasts and inhibits osteoclastogenesis leading to bone loss prevention [23]. One of the symptoms of menopaused women is lower estrogen level causing menopause to occur [24]. To make sure that the animals were indeed ovariectomized/osteoporotic we measured the estradiol level in serum after 15 weeks of treatment. Estradiol levels in the OVX Veh group were significantly lower than in Sham Veh (**Fig.2B**). No statistically significant difference in Estradiol level was observed between OVX Veh and OVX NMP groups (**Fig.2B**). Overall these parameters indicate the successful establishment of the estrogen deficient animal model.

3.3 Effect of NMP on serum bone markers

Fifteen weeks after OVX, analysis of bone markers were performed on serum by using ELISA kits for C-terminal cross-linked telopeptides of type I collagen (CTX-I), N-terminal propeptide of type 1 procollagen (P1NP) and Osteocalcin (OC).

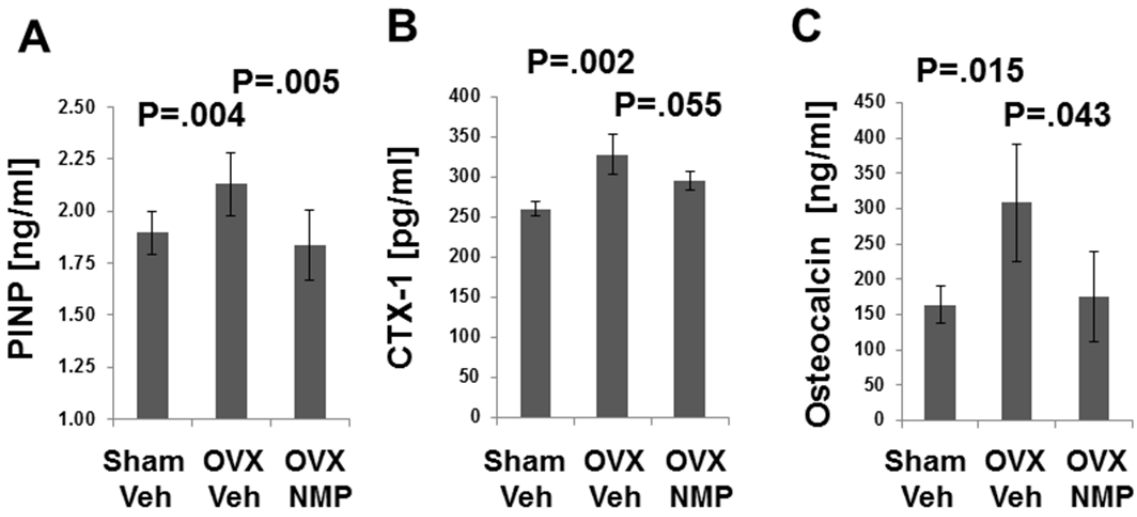

Figure 3: Changes of serum levels of bone markers after ovariectomy: Animals had blood drawn and measurements of bone markers in serum were performed. (A) Change of PINP (B) change of CTX-1 and (C) change of Osteocalcin levels in serum. The results of the Sham group were compared with OVX Veh group ($p<0.05$), and the results of the OVX NMP group were compared with the results of OVX Veh ($P<0.05$). Data are mean ± SD.

We found that the levels of all bone markers were higher in OVX Veh group compared with those in Sham Veh group (**Fig.3**). NMP treatment significantly lowered the CTX-I and P1NP induced by OVX. Similarly, the increase of OC serum level induced by OVX was prevented by treatment with NMP.

3.4 Effects of NMP on bone parameters

Dynamic histomorphometry analysis revealed that NMP had a significant effect on bone formation (**Fig.4**). The level of mineral apposition rate (MAR), a parameter that reflects individual osteoblast-mediated bone formation, [25] was significantly higher in Sham Veh compared to OVX Veh group (**Fig.4**). This was also apparent for

long bones (Supplemental figure 3). In NMP treated OVX group MAR increased significantly compared to OVX Veh group. However, the OVX NMP treated group did not reach the level of the Sham Veh group.

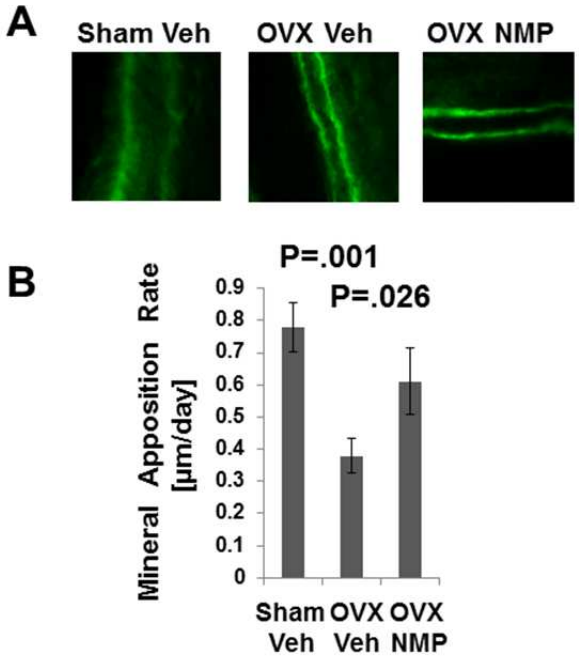

Figure 4: Dynamic histomorphometry analysis: Imaging of bone formation by fluorescence microscopy after calcein double labeling was performed. Two doses of calcein, a fluorochrome that incorporates at the site of newly formed bone mineralization, were injected to our animal model 10 days apart. The gap between the two green markers represents the amount of new bone formed during this time period. Figure 4A shows calcein double labeling under florescence microscope and Figure 4B demonstrates the mineral apposition rate (MAR). P values for (OVX Veh vs Sham Veh), and (OVX NMP vs OVX Veh) are provided in the figure.

Static histomorphometry on longitudinal sections of femur was also done with hematoxylin and eosin (H&E), and Goldner's Trichrome stain (Fig.5). Bone in the metaphysis region, right under the growth plate was analyzed and compared between different animal groups (magnification × 20). The results support what was already observed by the bone markers that NMP treatment prevents bone loss in OVX-treated group compared to the control group.

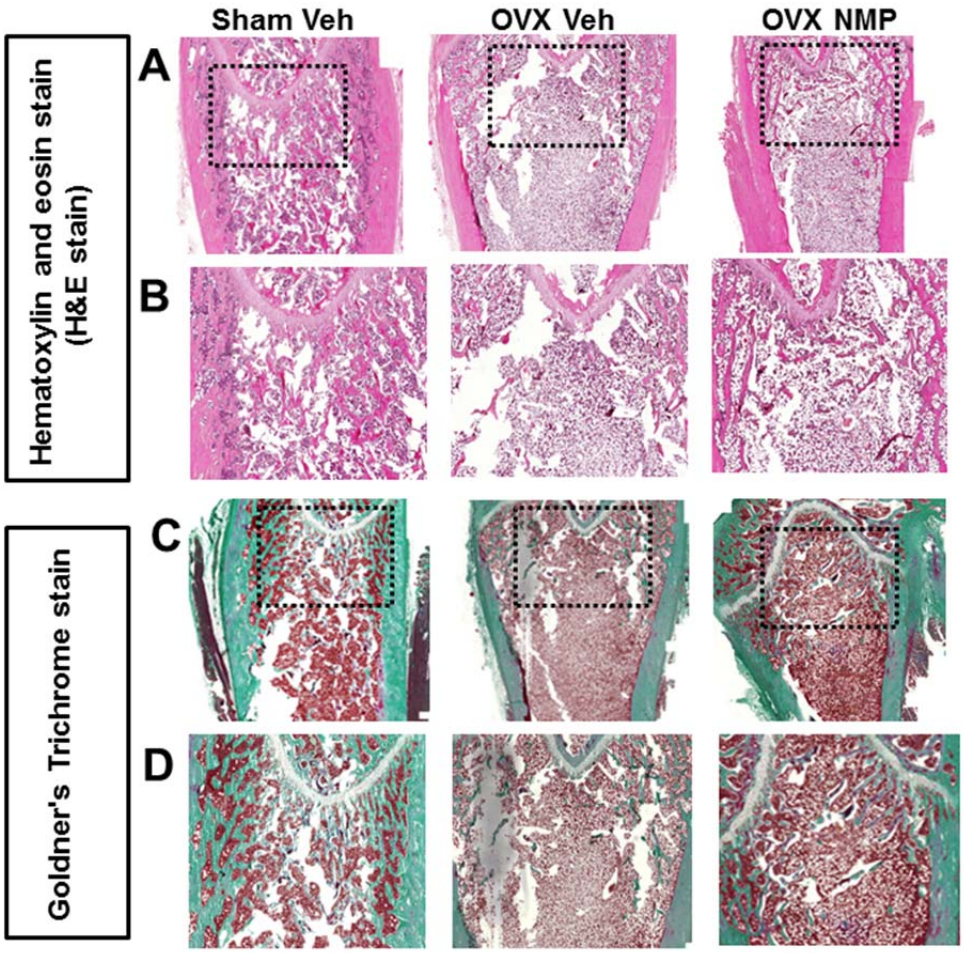

Figure 5: Static histomorphometry: Analyses of rat femur were observed under the microscope after specific histomorphometric staining (magnification x 20). Femoral paraffin sections from rats were stained with Hematoxylin and Eosin and Goldner's Trichrome to visualize and compare bone surfaces between the groups. Sections stained with H&E show a clear indication of denser trabecular bone in both Sham and OVX treated group compared to OVX Veh (A) and Goldner's trichrome staining, in which mineralized bone matrix was stained green while bone marrow and erythrocytes were stained orange/red. In Figure 5B the number of trabeculae in OVX Veh was noticeably less allowing for expansion of marrow and adipocytes (white gaps). The dotted squares (panel A, C) indicate the area of the histology seen in the picture below.

3.5 Micro-computed tomography (μCT)

Three-dimensional reconstruction images of the femurs showed differences in trabecular microarchitecture among the groups as represented in **Figure 6**. Analysis of different samples indicated that OVX resulted in the deterioration of the trabecular bone microarchitecture, as demonstrated by the reduced BV/TV and Tb.N compared

with the sham group. In contrast, Tb.Sp was significantly increased in response to OVX compared to the Sham group. NMP treatment significantly improved the microarchitecture deterioration mentioned above, but NMP was not able to reverse these parameters to a similar degree as in the Sham Veh group. Cortical bone parameters are not significantly different between groups (Supplemental figure 4).

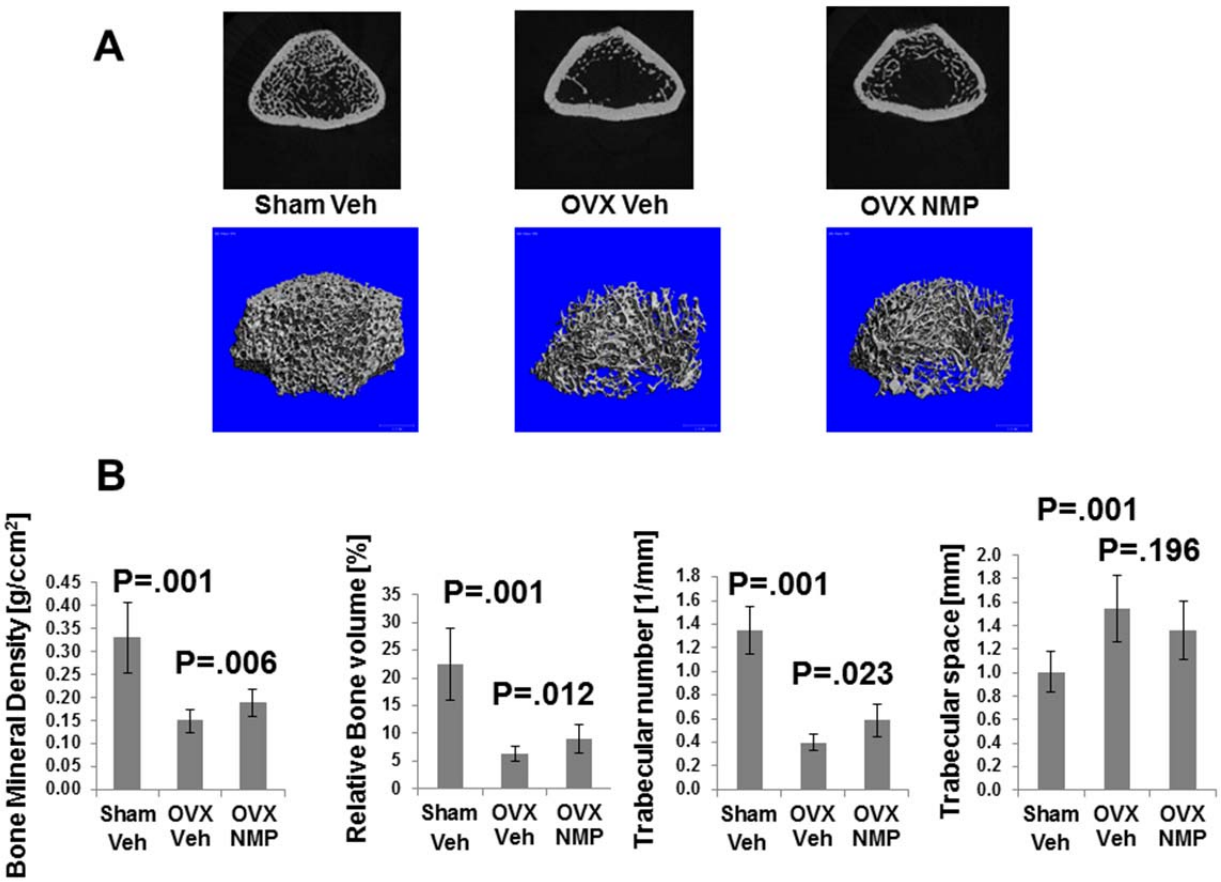

Figure 6: Representative Micro-CT images of trabecular bone microarchitecture in the distal femurs: After 15 weeks of treatment femurs from the OVX rats were assessed by microCT for different morphometric parameters. OVX Veh animals presented notable reduction in the trabecular number, trabecular area, bone mineral density and bone volume compared with Sham Veh and OVX NMP rats. Figure 6A represents the 3D reconstruction images of VOI in femurs and Figure 6B shows the morphometric bone parameters where the bar graphs represent the mean ± SD. P values (Sham Veh versus OVX Veh) and (OVX Veh versus OVX NMP) are provided in the figure.

4. Discussion

Bromodomains (BRDs) are small protein domains found in a variety of proteins which recognize and bind to acetylated histone tails. This specific binding action affects chromatin structure and helps the localization of transcriptional complexes to specific genes, consequently regulating epigenetically controlled processes including gene transcription. Inhibitors of the bromodomain and extra-terminal (BET) proteins, BRD and BRDT, which prevent bromodomain binding to acetyl-modified histone tails, have shown therapeutic promise in several diseases [26].

Recently, we showed that N-methyl pyrrolidone (NMP), a small water soluble molecule plays a significant role in the osteoblast and osteoclast differentiation [18, 19]. In the present study we describe the possible role of NMP as a clinically applicable bromodomain inhibitor and provide for the first time evidence that NMP administered systematically prevents progressive bone loss typically induced in osteoporosis. Firstly, NMP was tested on BET family bromodomains to determine its affinity towards all BRD subtypes. The study of the impact of NMP on the binding capacity of different recombinant human bromodomains and BET showed that this small compound is able to inhibit the binding of BRD2 and BRD4 by 70-80%. However, the affinity of NMP (milliM range) is much lower than a known selective small-molecule bromodomain inhibitor, JQ1 (microM range). Due to this characteristic, NMP can be considered a low affinity bromodomain inhibitor. These results are consistent with the recently published data showing that NMP is a functional acetyl lysine mimetic molecule [17, 27].

In many studies, treatment of cells with JQ1 results in cell viability reduction and in general inhibition of transcription [28-30]. In contrast, low affinity of NMP appears to be less toxic and more beneficial in our system. Indeed, treatment with NMP enhances the effect of BMP2 on ALP activity, whereas treatment with JQ1 causes inhibition of BMP2 induced ALP activity. This suggests that the effect of NMP

on osteoblast differentiation resulting in a partial inhibition of bromodomain proteins, and the complete inhibition as that induced by JQ1 would be deleterious to the effect of BMP2 on osteoblastic differentiation. Thus, the high affinity bromodomain inhibitor appears not suitable in prevention of osteoporosis, since it inhibits not only osteoclast formation but also osteoblast maturation and activity. The low affinity bromodomain inhibitor NMP, however, inhibits osteoclast activity at the same concentration as it binds to bromodomains of the BET-protein family, enhances osteoblast activity and maturation [18, 19].

On the molecular level JQ1 binds from the BET-protein family predominantly to BRD4 [31]. BRD4 has been shown to be depleted from the RUNX2 loci [16] a transcription factor involved in the early phase of osteoblast differentiation. NMP by its affinity to BRD4 will certainly also interfere with the RUNX2 loci but it can apparently overcome this effect on the differentiation of osteoblasts via the enhancement of the kinase activity of the BMP-BMP-receptor complex for Smad and p38, since osterix, a later osteoblast transcription factor is increased significantly [19]. This is in line with BMP2 signaling via Smad to induce dlx5 transcription and via p38 to induce dlx5 phosphorylation. The phosphorylated dlx5 together with p300 binds to the promoter region of osterix to set on osterix transcription [32]. Therefore, the enhanced phosphorylation of Smad and p38 by NMP [19] is sufficient for an increase in osterix phosphorylation independent of Runx2 transcription which is most likely decreased by the binding of NMP to the bromodomain of Brd4 (Fig. 1).

Moreover, JQ1 also inhibits osteoclast differentiation by interfering with BRD4-dependent RANKL activation of NFATC1 transcription [16]. NMP was also shown to inhibit RANKL activation of NFATC1 transcription [18] most likely in a BRD4 dependent fashion enhanced by blocking ERK phosphorylation which reduces cFos

transcription and AP-1 activation and with it an additional pathway for the induction of NFATC1 transcription.

Due to the *in vitro* effect of NMP on bone remodeling, we decided to evaluate its potential anti-osteoporotic effect on estrogen-deficiency induced osteoporosis. The ovariectomized rat model is a well-established model for estrogen-deficiency induced bone loss and has been previously used by a large number of researchers to test drugs for osteoporosis treatment and prevention [33-35].

It is well known that estrogen deficiency induces body fat accumulation and subsequently causes an increase in body weight [36]. Heine *et al.* demonstrated that estrogen receptor (ER) knockout mice have higher fat mass and lower energy expenditure than wild-type mice [37]. Estrogen may be involved directly in energy metabolism by binding to the ER within the abdominal and subcutaneous fat tissue [38]. In our experiments, subcutaneous administration of NMP did not significantly affect serum estradiol concentration. However, NMP did lower body weight gain in OVX rats. These results suggest that, NMP might have anti-osteoporotic effects in OVX rats, without the influence on hormone levels such as from estrogen.

Bone loss after ovariectomy is associated with high bone turnover where bone resorption exceeds bone formation. Subcutaneous injection of NMP significantly decreased CTX-1, and OC levels compared to an OVX-control group, suggesting that NMP decreased bone turnover induced by OVX. Furthermore, dynamic histomorphometry analysis shows that NMP increases new bone formation.

The most important parameter related to fracture risk is the mechanical strength of bones. The composition of long bones differs depending on the area. The midshaft region is dominated by cortical bone whereas the proximal femur is dominated by cancellous bone [39]. The effect of menopause or ovariectomy is more

pronounced on cancellous than cortical bone [40]. Due to the limited examination time of 15 weeks we mainly saw changes in cancellous bone. One could speculate that over extended time periods we might have seen OVX induced cortical bone destruction and preservation with NMP, since advanced postmenopausal women with osteopenia and fractures show significant effect also in the cortical bone [41].

In the present study, we established that NMP treatment effectively protected against loss of bone density by estrogen depletion. Since BMD has been described as only a substitute measure of bone strength, [42] microarchitecture determinants are necessary to evaluate the real impact of a treatment on trabecular bone quality. Femoral microarchitecture analyzed by micro-CT revealed that NMP treatment could prevent deterioration of the bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and decrease trabecular space (Tb.Sp) parameters compared to the OVX Veh control. The results suggested a moderate but significant effect of NMP on trabecular microarchitectural properties preservation. These findings are in agreement with other authors and drugs that were unable to completely restore the trabecular bone structure after its deterioration had occurred, emphasizing the need for prevention of trabecular bone loss. It has been suggested that bone resorption is increased with oxidative stress by promoting osteoclastic differentiation. Also, the toxicity of NMP must be evaluated before it can be used in the development of a drug or supplement. In this study, subcutaneous injection of NMP caused no changes that could be considered toxicologically significant.

In conclusion our results show that NMP, a nontoxic, inexpensive compound already used as a constituent in FDA-approved medical devices, possesses significant anti-osteoporotic activity in OVX rats. It improved both mass and quality of bones in OVX rat. The bone sparing action of NMP may be associated with its characteristic as a low affinity bromodomain inhibitor in conjunction with the enhancement of Smad and

p38 phosphorylation in osteoblasts to overcome the inhibition of osteoblast differentiation and inhibition of ERK phosphorylation in preosteoclasts to enhance the inhibition of osteoclast differentiation even further. Since NMP influences the osteoblasts/osteoclasts balance *in vitro* and shows an anti-osteoporotic effect *in vivo*, it can help postmenopausal women from losing bone mass. Overall, NMP seems to be a potential candidate for the application of epigenetics as new mechanism for osteoporosis treatment without any serious side effects.

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6. Appendix A. Supplementary data

Supplementary data to this article can be found online at http://

7. References

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